Example 9

Plutella xylostella (Diamondback Moth)

A. Cloning of a Partial Sequence of the *Plutella xylostella*

[0427] High quality, intact RNA was isolated from all the different larval stages of *Plutella xylostella* (Diamondback moth; source: Dr. Lara Senior, Insect Investigations Ltd., Capital Business Park, Wentloog, Cardiff, CF3 2PX, Wales, UK) using TRIzol Reagent (Cat. Nr. 15596-026/15596-018, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions. Genomic DNA present in the RNA preparation was removed by DNase treatment following the manufacturer's instructions (Cat. Nr. 1700, Promega). cDNA was generated using a commercially available kit (SuperScript™ III Reverse Transcriptase, Cat. Nr. 18080044, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions.

[0428] To isolate cDNA sequences comprising a portion of the PX001, PX009, PX010, PX015, PX016 genes, a series of PCR reactions with degenerate primers were performed using Amplitaq Gold (Cat. Nr. N8080240, Applied Biosystems) following the manufacturer's instructions.

[0429] The sequences of the degenerate primers used for amplification of each of the genes are given in Table 2-PX. These primers were used in respective PCR reactions with the following conditions: 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 50° C. and 1 minute and 30 seconds at 72° C., followed by 7 minutes at 72° C. (for PX001, PX009, PX015, PX016); 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 54° C. and 2 minute and 30 seconds at 72° C., followed by 7 minutes at 72° C. (for PX010). The resulting PCR fragments were analyzed on agarose gel, purified (QIAquick Gel Extraction kit, Cat. Nr. 28706, Qiagen), cloned into the pCR8/GW/TOPO vector (Cat. Nr. K2500-20, Invitrogen) and sequenced. The sequences of the resulting PCR products are represented by the respective SEQ ID NO:s as given in Table 2-PX and are referred to as the partial sequences. The corresponding partial amino acid sequence are represented by the respective SEQ ID NO:s as given in Table 3-PX.

B. dsRNA Production of the *Plutella xylostella* Genes

[0430] dsRNA was synthesized in milligram amounts using the commercially available kit T7 Ribomax™ Express RNAi System (Cat. Nr. P1700, Promega). First two separate single 5' T7 RNA polymerase promoter templates were generated in two separate PCR reactions, each reaction containing the target sequence in a different orientation relative to the T7 promoter.

[0431] For each of the target genes, the sense T7 template was generated using specific T7 forward and specific reverse primers. The sequences of the respective primers for amplifying the sense template for each of the target genes are given in Table 8-PX. The conditions in the PCR reactions were as follows: 1 minute at 95° C., followed by 20 cycles of 30 seconds at 95° C., 30 seconds at 60° C. (−0.5° C./cycle) and 1 minute at 72° C., followed by 15 cycles of 30 seconds at 95° C., 30 seconds at 50° C. and 1 minute at 72° C., followed by 10 minutes at 72° C. The anti-sense T7 template was generated using specific forward and specific T7 reverse primers in a PCR reaction with the same conditions as described above. The sequences of the respective primers for amplifying the anti-sense template for each of the target genes are given in Table 8-PX. The resulting PCR products were analyzed on agarose gel and purified by PCR purification kit (Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and $NaClO_4$ precipitation. The generated T7 forward and reverse templates were mixed to be transcribed and the resulting RNA strands were annealed, DNase and RNase treated, and purified by sodium acetate, following the manufacturer's instructions. The sense strand of the resulting dsRNA for each of the target genes is given in Table 8-PX.

C. Laboratory Trials to Test dsRNA Targets, Using Artificial Diet for Activity Against *Plutella xylostella* Larvae

[0432] Diamond-back moths, *Plutella xylostella*, were maintained at Insect Investigations Ltd. (origin: Newcastle University, Newcastle-upon-Tyne, UK). The insects were reared on cabbage leaves. First instar, mixed sex larvae (approximately 1 day old) were selected for use in the trial. Insects were maintained in Eppendorf tubes (1.5 ml capacity). Commercially available Diamond-back moth diet (Bio-Serv, NJ, USA), prepared following the manafacturer's instructions, was placed in the lid of each tube (0.25 ml capacity, 8 mm diameter). While still liquid, the diet was smoother over to remove excess and produce an even surface.

[0433] Once the diet has set the test formulations are applied to the diet's surface, at the rate of 25 μl undiluted formulation (1 μg/μl dsRNA of targets) per replicate. The test formulations are allowed to dry and one first instar moth larva is placed in each tube. The larva is placed on the surface of the diet in the lid and the tube carefully closed. The tubes are stored upside down, on their lids such that each larva remains on the surface of the diet. Twice weekly the larvae are transferred to new Eppendorf tubes with fresh diet. The insects are provided with treated diet for the first two weeks of the trial and thereafter with untreated diet.

[0434] Assessments are made twice weekly for a total of 38 days at which point all larvae are dead. At each assessment the insects are assessed as live or dead and examined for abnormalities. Forty single larva replicates are performed for each of the treatments. During the trial the test conditions are 23 to 26° C. and 50 to 65% relative humidity, with a 16:8 hour light:dark photoperiod.

D. Cloning of a DBM Gene Fragment in a Vector Suitable for Bacterial Production of Insect-Active Double-Stranded RNA

[0435] What follows is an example of cloning a DNA fragment corresponding to a DBM gene target in a vector for the expression of double-stranded RNA in a bacterial host, although any vector comprising a T7 promoter or any other promoter for efficient transcription in bacteria, may be used (reference to WO0001846).

[0436] The sequences of the specific primers used for the amplification of target genes are provided in Table 8-PX. The template used is the pCR8/GW/topo vector containing any of target sequences. The primers are used in a PCR reaction with the following conditions: 5 minutes at 98° C., followed by 30 cycles of 10 seconds at 98° C., 30 seconds at 55° C. and 2 minutes at 72° C., followed by 10 minutes at 72° C. The resulting PCR fragment is analyzed on agarose gel, purified